

Correspondence

## DNA segments of African Swine Fever Virus detected for the first time in hard ticks from sheep and bovines

ZE CHEN<sup>1</sup>, XIAOFENG XU<sup>1</sup>, YUFENG WANG<sup>2</sup>, JINLONG BEI<sup>3</sup>, XIUFENG JIN<sup>1</sup>, WEIHAO DOU<sup>1</sup>, HAISHUO JI<sup>1</sup>, YUJIE DUAN<sup>1</sup>, XIAOJUN YANG<sup>2\*</sup> & SHAN GAO<sup>1\*</sup>

<sup>1</sup>College of Life Sciences, Nankai University, Tianjin 300071, P.R. China.

<sup>2</sup>Southwest Forestry University, Kunming 650224, P.R. China.

<sup>3</sup>AGRO-Biological Gene Research Center, Guangdong Academy of Agricultural Science, Guangzhou 510640, P.R. China.

\*Correspondence to: Shan Gao E-mail: gao\_shan@mail.nankai.edu.cn (SG); Xiaojun Yang E-mail: yangxj86@163.com

### ABSTRACT

In this study, we detected African Swine Fever Virus (ASFV) in *Dermacentor* (Ixodidae) from sheep and bovines using small RNA sequencing. To validate this result, a 235-bp DNA segment was detected in a number of DNA samples from *D. silvarum* and sheep blood. This 235-bp segment had an identity of 99% to a 235-bp DNA segment of ASFV and contained three single nucleotide mutations (C38T, C76T and A108C). C38T, resulting in a single amino acid mutation G66D, suggests the existence of a new ASFV strain, which is different from all reported ASFV strains in the NCBI GenBank database and the ASFV strain (GenBank: MH713612.1) reported in China in 2018. To further confirm the existence of ASFV in *Dermacentor* ticks, three DNA segments of ASFV were detected in *D. niveus* females from bovines and their first generation ticks reared in our lab. These results also proved that transovarian transmission of ASFV occurs in hard ticks. This study revealed for the first time that ASFV has a wider range of hosts (e.g. sheep and bovines) and vectors (e.g. hard ticks), beyond the well-known Suidae family and Argasidae (soft ticks). Our findings pave the way toward further studies on ASFV transmission and the development of prevention and control measures.

**KEYWORDS:** ASFV; hard tick; virus detection; transmission; transovarian

African Swine Fever Virus (ASFV) is a large (~ 190 kbp), double-stranded DNA virus with a linear genome containing at least 150 genes. ASFV causes African Swine Fever (ASF), a highly contagious viral disease of swine. ASF results in high mortality, approaching 100% (Galindo & Alonso 2017). ASF first broke out in Africa in the early 1900s (Montgomery 1921) and remained restricted to Africa until 1957, when it was reported in Portugal. In the following years, ASF spread further geographically and caused economic losses in the swine industry. In 2018, ASF was reported in the Liaoning province of China and then spread to 20 provinces and 4 municipalities. Although ASFV can be quickly detected using PCR with specific primers (Yang *et al.* 2011), the understanding of ASFV hosts and vectors is still limited to the Suidae family and soft ticks, respectively (Chen *et al.* 2011).

It is well accepted that ASFV infects members of the Suidae family, including domestic pigs, warthogs, and bushpigs (Denyer & Wilkinson 1998) and soft ticks belonging to the genus *Ornithodoros* are actual biological vectors of ASFV. In China, only 19 species of Ixodidae (including *D. nuttalli*, *D. silvarum*, *Amblyomma testudinarium* and so on) have been reported infesting Suidae (Chen *et al.* 2019). However, to date, all of these tick species have never been recorded as ASFV vectors (Chen *et al.*, 2011; Yu *et al.*, 2015). To the best of our knowledge, many *Dermacentor* ticks share similarities to *Ornithodoros* ticks in their range of hosts, but *Dermacentor*

ticks have higher mobility and a wider geographical distribution. Compared to *Ornithodoros* and other Ixodidae ticks, *A. testudinarium* has a wider geographical distribution in the south of China, where ASF has been reported in 2018, whereas *Ornithodoros* occurrences have not been documented. *A. testudinarium* also has a wider range of hosts covering all members of the Suidae family. In addition, *Dermacentor* and *Amblyomma* ticks produce much more eggs than *Ornithodoros* ticks. The viruses transmitted by *D. nuttalli*, *D. silvarum*, and *A. testudinarium* are under-estimated in terms of those transmitted by insects, based on our previous studies using high-throughput sequencing (Zheng *et al.* 2017).

Small RNA sequencing (small RNA-seq or sRNA-seq) is used to obtain thousands of short RNA sequences with lengths that are usually less than 50 bp. sRNA-seq has been successfully used for virus detection in plants (Li *et al.* 2012), invertebrates and humans (Wang *et al.* 2016). In 2016, an automated bioinformatics pipeline, VirusDetect, reportedly facilitated large-scale virus detection using sRNA-seq (Zheng *et al.* 2017). This study aimed to detect viruses in *Dermacentor* ticks using sRNA-seq. Unexpectedly, VirusDetect reported the existence of ASFV in *D. nuttalli* from sheep and bovines. To confirm this result, a 235-bp DNA segment of ASFV (AY261365.1:103819-104053) was detected in *D. nuttalli*, *D. silvarum* and sheep, but not in *A. testudinarium* and bovines. To further confirm the existence of ASFV in *Dermacentor* ticks, three DNA segments of ASFV were detected in *D. niveus* females from bovines and their first generation (F1) ticks reared in our lab.

*Amblyomma testudinarium* ticks were captured from buffalo in Yunnan province of China. *D. nuttalli*, *D. silvarum* and *D. niveus* ticks were captured from sheep and bovines in Xinjiang Autonomous Region of China. Several ticks were used to pool four samples representing *A. testudinarium* adults, *D. nuttalli* adults, *D. niveus* larvae and *D. niveus* nymphs to construct four sRNA-seq libraries, which were sequenced using 50-bp single-end strategy on the Illumina HiSeq 2500 platform, respectively (Chen *et al.* 2017). As the library of *D. nuttalli* adults was sequenced twice, five runs of sRNA-seq data were deposited at the NCBI SRA database under the project accession number SRP084097 and SRP178347.

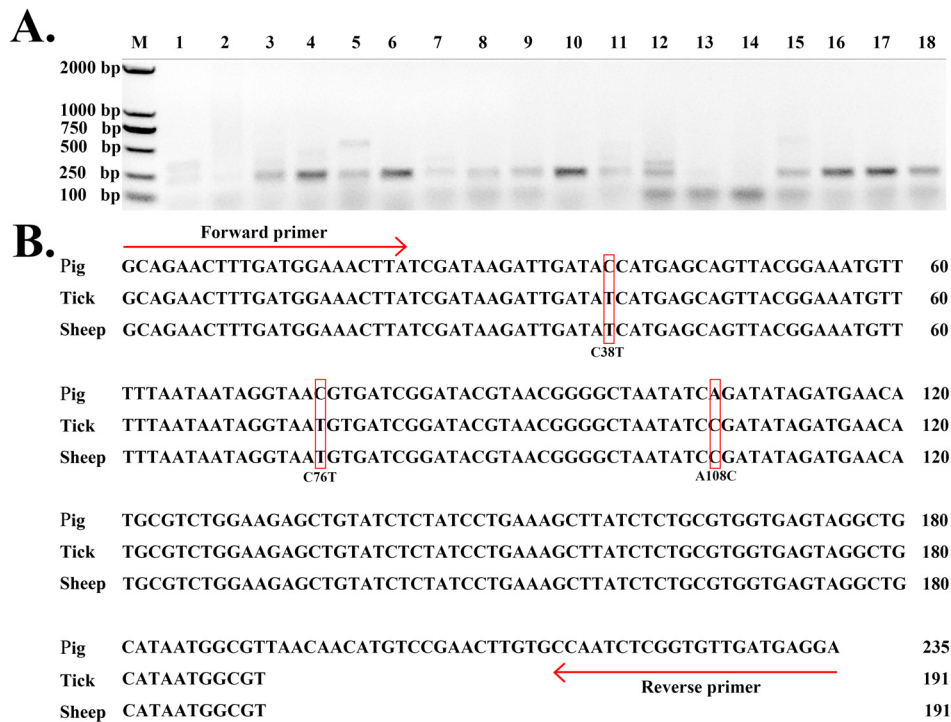
The cleaning and quality control of sRNA-seq data were performed using the pipeline Fastq\_clean (Zhang *et al.* 2014) that was optimized to clean the raw reads from Illumina platforms. The virus detection was performed using the pipeline VirusDetect (Zheng *et al.* 2017). The detection of siRNA duplexes was performed using the program duplexfinder (Niu *et al.* 2017). Statistical computation and plotting were performed using the software R v2.15.3 with the Bioconductor packages (Gao *et al.* 2014). The ASFV reference genome (GenBank: AY261365.1) was used for all the data analysis in this study.

The RNA extraction of *D. nuttalli* ticks and the cDNA synthesis were performed using the protocol published in our previous study (Gao *et al.* 2018). The DNA extraction of *D. nuttalli*, *D. silvarum*, *D. niveus* ticks, sheep and bovine blood was performed using the protocol published in our previous study (Cheng *et al.* 2017). PCR reaction was performed using Thermo Scientific DreamTaq Green PCR Master Mix (2×) by incubation at 95 °C for 3 min, followed by 34 PCR cycles (30 s at 95 °C, 30 s at 55 °C, and 60 s at 72 °C for each cycle) and a final extension at 72 °C for 5 min. The primers to amplify three DNA segments of ASFV are listed in **Table 1**.

After data cleaning and quality control, 13,496,191, 25,194,632, 37,888,277, 12,302,335, and 15,077,054 clean reads were used to detect viruses in *A. testudinarium* adults, *D. nuttalli* adults, *D. nuttalli* adults (replicate), *D. niveus* larvae, and *D. niveus* nymphs. VirusDetect reported the existence of ASFV in all four *Dermacentor* samples, but not in the *A. testudinarium* sample. VirusDetect uses the closest reference sequence to report the detected virus. The closest reference genome (GenBank: AY261365.1) of ASFV was sequenced using ASFV in ticks captured in Warmbaths of South Africa in a previous study. Aligning the clean reads to the ASFV reference genome, the mapping rates of four *Dermacentor* samples reached 0.06% (15,585/25,194,632),

0.06% (23,330/37,888,277), 0.08% (10,241/12,302,335), and 0.08% (12,807/15,077,054), which were significantly higher than the mapping rate of 0.01% (905/13,496,191) from the *A. testudinarium* sample. The length distribution of virus derived small RNAs (vsRNAs) in four *Dermacentor* samples was concentrated to 15–19 bp rather than 21–24 bp. The length distribution of vsRNAs was different from those in plants vsRNAs (Niu *et al.* 2017) and other invertebrate vsRNAs (Liu *et al.* 2018). We did not detect small interfering RNA (siRNA) duplexes from ASFV in four *Dermacentor* samples.

To validate the existence of ASFV, we used PCR with specific primers to amplify a 235-bp ASFV segment (AY261365.1:103819-104053) using total RNA of the *D. nuttalli* adults, from which ASFV had been detected using sRNA-seq. Gel electrophoresis depicted a clear 235-bp band as was expected, however Sanger sequencing failed due to the low cDNA concentration. To confirm these results, we used PCR to detect this segment in a large number of DNA samples from *D. silvarum* ticks, sheep and bovine blood. Gel electrophoresis showed that 235-bp bands appeared in 33 and 12, out of 80 *D. silvarum* samples and 100 sheep blood samples, respectively (**Figure 1A**). Two 235-bp DNA sequences were obtained using Sanger sequencing from *D. silvarum* and sheep, to confirm that these bands represented the 235-bp ASFV segment (**Figure 1B**). In addition, gel electrophoresis showed 560-bp bands in 41 out of 100 bovine samples. We then obtained two 560-bp sequences using Sanger sequencing. Using the Basic Local Alignment Search Tool (BLAST), we inferred that these 560-bp bands could be segments from the bovine genome.



**FIGURE 1.** 235-bp DNA segments of ASFV in hard ticks and sheep. **A.** Lanes 1–9 used DNA from *D. silvarum* and Lanes 10–18 used DNA from sheep blood. **B.** “Pig” represents the 235-bp DNA segment of the ASFV reference genome (GenBank: AY261365.1). “Tick” represents the 235-bp DNA segment from *D. silvarum*. “Sheep” represents the 235-bp DNA segment from sheep blood. *D. silvarum* and sheep blood were collected from two different places in Xinjiang Autonomous Region of China.

Further analysis of two 235-bp sequences from *D. silvarum* and sheep showed an identity of 100% between them (**Figure 1B**). Using the ASFV reference genome (GenBank: AY261365.1), three single nucleotide mutations were detected at positions 38, 76, and 108 (C38T, C76T and A108C) on these two 235-bp sequences. As the 235-bp ASFV segment encodes 78 amino acids, C38T results in a single amino acid mutation at position 66 (G66D). C38T suggested the existence of a new ASFV strain, which was different from all reported ASFV strains in the NCBI GenBank database (version 197) and the ASFV strain (GenBank: MH713612.1) reported in China in 2018. BLAST confirmed that this newly detected 235-bp segment in the new ASFV strain did not have high similarity with sequences from other viruses, bacteria or animal genomes. Therefore, this segment was highly specific to ASFV. The above results proved the existence of ASFV in *D. nuttalli*, *D. silvarum* and sheep. As *D. silvarum* ticks and sheep blood were collected from two different places, this new strain could infect sheep and be transmitted by *D. silvarum* ticks.

To further confirm the existence of ASFV in *Dermacentor* ticks, we reared *D. niveus* females from bovines using New Zealand white rabbits. The rabbits were maintained at 18–20°C with 50% relative humidity (RH), and exposed to natural daylight cycles. After detachment, ticks were collected and incubated in cotton-plugged glass tubes filled with one folded filter paper in an incubator [(20 ± 1°C), 50% RH, natural daylight cycles]. Then, we used PCR to amplify the 235-bp ASFV segment (AY261365.1:103819-104053), a 257-bp ASFV segment (AY261365.1:105122-105378) and a 322-bp ASFV segment (AY261365.1:124995-125316) in *D. niveus* females and their F1 generation ticks. Sanger sequencing proved the existence of ASFV and the occurrence of transovarian transmission of ASFV in *D. niveus*. Using the ASFV reference genome (GenBank: AY261365.1), we identified a total of 10 single nucleotide mutations and three single amino acid mutations from these three segments. These results suggested the existence of another new ASFV strain, which could infect bovines and be transmitted by *D. niveus* ticks.

In conclusion, we detected a 235-bp ASFV segment in hard ticks from sheep and bovines, and proved the existence of a new ASFV strain, which is different from all reported ASFV strains in the Suidae family and soft ticks. As two 235-bp sequences from *D. silvarum* and sheep showed an identity of 100% between them, the genomes of ASFV in *D. silvarum* and sheep need to be sequenced in order to determine their identity. Further experiments and analysis proved the existence of ASFV and the occurrence of transovarian transmission of ASFV in *D. niveus*. This suggested the existence of another new ASFV strain. Besides providing a basis for future research, we also provide *D. niveus* ticks (reared in our lab) for use in future studies on ASFV transmission and the development of prevention and control measures.

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**TABLE 1.** Primers to amplify three DNA segments of ASFV.

No.	Location	Forward Primer	Reverse Primer
1	103819-104053	GCAGAACTTTGATGGAACTTA	TCCTCATCAACACCGAGATTGGCAC
2	105122-105378	CCCTGAATCGGAGCATCCT	AGTTATGGGAAACCCGACCC
3	124995-125316	AAGGAGGATGTGCATTTCATT	AGGTCATCTTCAAAACGGAT

Three DNA segments are located on the ASFV reference genome (GenBank: AY261365.1). The first and second segments belong to the capsid protein p72 gene. The third segment belongs to the helicase superfamily II group gene.

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